



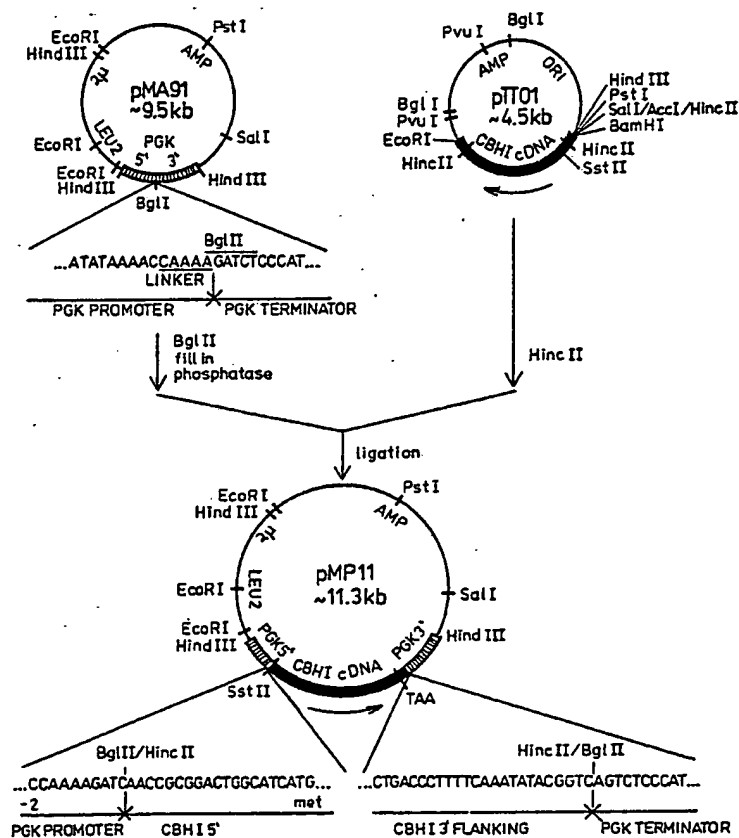
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(54) Title: YEAST STRAINS PRODUCING CELLULOLYTIC ENZYMES AND METHODS AND MEANS FOR CONSTRUCTING THEM

(57) Abstract

Methods and means for the construction of strains of yeast capable of producing cellulolytic enzymes. This is achieved by the transfer of chromosomal genes or cDNA copies of mRNAs coding for cellulolytic enzymes isolated from the fungus *Trichoderma reesei* to yeast cells using recombinant DNA vectors capable of replicating in yeast. The correct expression of these cellulolytic genes in yeast leads to the production of cellulolytic enzymes which are secreted from the cell. This allows the yeast to hydrolyse β -1,4-glucan substrates such as cellulose.



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YEAST STRAINS PRODUCING CELLULOLYTIC ENZYMES AND METHODS AND
MEANS FOR CONSTRUCTING THEM

BACKGROUND OF THE INVENTION

Three different classes of enzymatic activity have been shown to be required for the complete hydrolysis of cellulose to glucose. The two major activities involved in cellulose solubilization are endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91) (1, 2). For the production of glucose a third type of activity, cellobiase or β -glucosidase (EC 3.2.1.21) is also required. The precise manner in which these three different classes of enzyme interact to bring about the complete hydrolysis of cellulose is not yet clear.

Same filamentous fungi produce a number of different isoenzymes of each class of cellulolytic enzyme which apparently interact synergistically in hydrolysis (3, 4, 5, 6).

Trichoderma has been shown to produce at least two immunologically distinct cellobiohydrolases CBH I and CBH II, at least 2 endoglucanases, ENDO II and ENDO III, and a β -glucosidase. While enzymatic hydrolysis of cellulose proceeds most rapidly in the presence of all these enzymes, CBH I alone is able to degrade crystalline cellulose to glucose and cellobiose (7, 8, 9).

Two groups have reported the molecular cloning of the T. reesei gene for CBH I and the complete sequence of this gene is known (10, 11).

Yeast is an important industrial organism and is used for brewing, wine making, baking, ethanol production, single cell protein production and more recently for the production of pharmaceuticals such as interferon, growth hormone and Hepatitis B virus antigen. Yeast do not produce enzymes that degrade cellulose. The development of yeast strains able to hydrolyse cellulose would make possible improvements in existing processes

where cellulose or glucans are present in the raw material used. As important would be the possibility of developing new processes not currently possible.

In filtration and clarification of beer high molecular weight β -glucans originating from barley grain cause problems. In the brewing industry microbial β -glucanases are used to remove these β -glucans. If the yeast used in the production of beer were able to produce endoglucanases, the filterability of beer would significantly be improved and the cost of filtering would decrease. By transferring individual fungal cellulase genes to yeast it is possible to produce yeast strains that produce only one cellulase enzyme. Such yeast strains would produce enzymes for use in, for example the pulp and paper industry. Cellulose used in paper making could be swelled by pretreating with one cellulase enzyme, which would bring about swelling without excessive hydrolysis of cellulose.

There are two ways in which a foreign gene can be expressed in yeast. The simplest is to join the whole gene from the chromosome of the donor organism to a yeast vector and transform a yeast cell. If the yeast genetic system recognizes the appropriate sequences in the transferred gene the gene will be expressed. However, in practice this is rare and depends at least in part on the genetic distance between the donor organism and the yeast.

For example, of the five genes from Aspergillus niger tested in Saccharomyces cerevisiae, only one of these was found to express (12). Therefore it cannot be assumed that heterologous genes will automatically be expressed in yeast.

The second method of obtaining expression of genes in yeast is by connecting either the chromosomal gene or a cDNA copy of the messenger RNA coding for the desired gene to a yeast promotor sequence. In this way, human eukaryote interferon (13), hepatitis B virus surface antigen (14), bovine rennin (15), and mouse α -amylase (16) have all been expressed in yeast.

These and other studies show that while expression of the cDNA or gene is always obtained, the amount and cellular location of the product is very difficult to predict in the absence of experimentation. Montenecourt (1) outlined a number of possible cloning strategies for cloning cellulase genes from T. reesei but did not describe the methods to be used to achieve the goal.

SUMMARY OF THE INVENTION

In accordance with this invention described are yeast strains capable of producing cellulolytic enzymes, methods for construction of these strains, recombinant DNA vectors needed in the construction of these strains, methods used in the construction of these vectors, and cDNA copies of cellulolytic enzymes coding genes.

Chromosomal genes coding for three different cellulases, CBH I, CBH II and ENDO II were isolated from a λ phage gene library of T. reesei by differential hybridisation. Fragments of these genes were used to isolate full length cDNAs from a T. reesei cDNA library.

cDNAs for the three cellulases CBH I, CBH II and ENDO II and the CBH I gene were transferred to suitable 2 μ yeast plasmids. When used to transform suitable yeast strains, they directed the expression and secretion of the respective cellulase enzyme. The cellulases produced by the yeast were shown to have similar activities to the native fungal enzyme.

A cellulolytic yeast strain *Saccharomyces cerevisiae* VTT-RC-84001 produced in accordance with the present invention has been deposited in the National Collection of Yeast Cultures, Norwich, United Kingdom, under the deposit number NCYC No. R 128 since April 6, 1984.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is described below in greater detail with reference to the accompanying drawings.

Figure 1 shows the restriction map of T. reesei cellobiohydrolase I (CBH I) chromosomal gene. The coding region is marked with thickened line.

Figure 2 shows the restriction map of T. reesei cellobiohydrolase II (CBH II) chromosomal gene. The coding region is marked with thickened line.

Figure 3 shows the restriction map of T. reesei endoglucanase II (ENDO II) chromosomal gene. The coding region is marked with thickened line.

Figure 4 shows the construction of plasmid YE_pNP03 for expression of a chromosomal copy of CBH I gene from T. reesei in yeast.

Figure 5 shows the cDNA sequence of the CBH II gene of T. reesei from plasmid pTT09.

Figure 6 shows the cDNA sequence of the ENDO II gene of T. reesei from plasmid pTT11. The positions of introns found in chromosomal copy of the gene are marked with arrow (+).

Figure 7 shows the construction of plasmid pMP11 for expression of T. reesei CBH I in yeast.

Figure 8 shows the construction of plasmid pMP29 for expression of T. reesei CBH II in yeast.

Figure 9 shows the construction of plasmid pMP311 for expression of T. reesei ENDO II in yeast.

Figure 10 shows the enzyme activity of CBH I produced by the yeast strain VTT-RC-84001.

Detailed description

The definitions used in this detailed description are as defined in the Gilbert and Talmadge Patent (USA) 4,338,397.

Materials

Bacterial and fungal strains, plasmids, and phage. T. reesei strain VTT-D-80133, a mutant strain with improved production of cellulolytic enzymes derived from QM 9414 (17) after several successive mutation steps (18), was used for isolation of the genes from cellobiohydrolase I (CBH I), cellobiohydrolase II (CBH II) and endoglucanase II (ENDO II).

Escherichia coli strains Q358 and Q359 and the phage λ 1059, used in the construction of the T. reesei gene bank were provided by Dr. J. Karn (19). E. coli HB 101 was used as a host in 5 transformation with the plasmid pBR 322. E. coli JM 101 and the phage M 13 mp 7 (20) and the plasmids pUC 8 and pUC 9 (21), used in the dideoxy sequencing, were from the laboratory of F. Sanger. Yeast strains used were Saccharomyces cerevisiae OL1 (Mata leu 2-3 leu 2-112 his 3-11 his 3-15 ura 3-251 ura 3-373) (22) and S. cerevisiae MT302-1c (Mata arg 5-6 leu 2-3 leu 2-112 his 3-11 his 3-15 pep 4-3 ade 1) (23).

A 12 kb cosmid p3030 obtained from Barbara Hohn, which replicates both in E. coli and in yeast was used as vector for transferring the chromosomal copy of CBH I to yeast. Cosmid p3030 contains genes for ampicillin and tetracycline resistance in E. coli and the his3 gene for selection in yeast. The vector contains a cos site which enables it to be packaged into infective λ phage particles in vitro and the yeast 2 μ EcoD fragment. Yeast

expression vector containing the phosphoglucocokinase (PGK) gene promoter was used for expression of the cDNA copies of cellulase genes in yeast (23).

Enzymes. Restriction enzymes were purchased from Amersham (UK), Boehringer Mannheim (FDR) and Bethesda Research Laboratories (Gaithersburg, MD) and used according to the manufacturers' instructions. T4 ligase and the DNA polymerase I large subunit were from Biolabs and the calf intestine phosphatase from Boehringer Mannheim. Reverse transcriptase was from Dr. J.W. Beard (Life Sciences Inc., St. Petersburg, Fla.). Protoplasting enzyme, Zymolyase 60000 was obtained from Kirin Brewery Co., Japan. Klenow fragment of E. coli polymerase I was from Boehringer Mannheim.

General growth media. E. coli HB101 was grown in L-broth. Transformants were selected on L-plates supplemented with 1.5 % agar and containing 100 µg/ml ampicillin. The concentration of tetracycline added to L-plates was 10 µg/ml. Complete medium YPG for growth of yeast contained 1 % yeast extract, 2 % peptone, and 2 % glucose. Yeast minimal medium, YMB, contained 0.67 % yeast nitrogen base (Difco, Detroit, USA) and 2 % sugar (lactose, cellobiose, starch or glucose). The final concentration of amino acids added was as described (24). The solidifying agent on yeast plates was 2 % agar (Difco Bacto Agar). In yeast protoplast plating medium 1.2 M sorbitol was added as an osmotic stabilizer. The top agar used in plating the yeast protoplasts for regeneration was prepared as minimal medium but using 3 % purified agar (Difco) as a solidifying agent.

All methods unless otherwise specified are as described in Maniatis et al. 1982 (25).

Isolation and characterization of the cellulolytic genes from the fungus *T. reesei*

Polyadenylated (polyA⁺) messenger RNA isolated from *T. reesei* mycelia actively producing cellulases directs in the in vitro synthesis - in a rabbit reticulocyte lysate - of a number of large polypeptides that are precipitated by antibody prepared against purified cellulolytic enzymes. Messenger RNA isolated from repressed glucose grown mycelia does not direct the synthesis of these cellulase-specific polypeptides. This difference between induced and repressed populations was used to identify a collection of hybrid λ phages containing *T. reesei* genes strongly expressed during production of cellulolytic enzymes.

For the isolation of cellulase-specific, induced mRNAs *T. reesei* (strain VTT-D-80133) was grown as described by Bailey and Nevalainen (26) except that the medium contained 2 % lactose and 2 % of a soluble extract of distillers spent grain. Samples taken during cultivation were assayed for activity against dyed Avicel, hydroxyethylcellulose (HEC) and for soluble protein (26). Estimation of reducing sugars was by the method of Sumner (27).

Cellular RNA from mycelia was isolated by a modification of the method of Ohi and Short (28). The frozen mycelia was ground to a fine powder under liquid nitrogen and suspended in a buffer containing 20 mM Tris-HCl (pH 7.6), 0.1 M NH₄⁺, 1 mM Mg (OAc)₂, 10 mM Na-iodoacetate, 0.5 mg/ml polyvinylsulfate and 2 % Na-dodecyl sulfate (SDS). Following incubation at 37 °C for 30 minutes, insoluble material was removed by centrifugation at 13000 g for 10 minutes.

The poly(A)⁺ fraction was purified by chromatography through an oligo(dT) cellulose column (Bethesda Research Laboratories (29) and in vitro translation was carried out with a rabbit reticulocyte lysate using ³⁵S-methionine (Amersham International Ltd) (30). Immunoprecipitation was carried out according to Dobber-

stein (31) using antiserum prepared against purified CBH I, CBH II or ENDO II, or with the corresponding preimmune serum.

Table 1 shows the molecular weights of proteins precipitated by antiserum against specific cellulases analysed on 7.5-15 % SDS polyacrylamide gels (32).

Table 1.

Antiserum	<u>In vivo</u>	<u>In vitro</u>
CBH I	71 000	67 000
CBH II	63 000	48 000
ENDO II	62 000	53 000

The construction of the T. reesei gene bank was carried out as follows.

Conidia of Trichoderma reesei were germinated in a liquid medium containing 1.5 % KH_2PO_4 , 0.5 % $(\text{NH}_4)_2\text{SO}_4$, 0.06 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06 % CaCl_2 , 0.15 % proteose peptone, 0.03 % urea, 2 % sucrose and minimal salts. Cultures were incubated with shaking at 29 °C for about 12 h. The isolation of nuclei was carried out using a slightly modified method of Hautala et al. (33). DNA was isolated from a crude nuclear pellet obtained by differential centrifugation of homogenized mycelium. The crude nuclear pellet was treated with SDS-amylase solution (100 mM EDTA pH 8.0, 140 mM NaCl, 1 % Nadecylsulfate and 3.3 % α -amylase obtained from Merck, Darmstadt, FRG) for 1 h at 37 °C. Proteinase K (final concentration 0.8 % w/v) was then added and incubation was continued for 2 h at 37 °C with gentle shaking. After incubation, cell debris was removed by centrifugation and DNA was precipitated from the supernatant with ethanol. The DNA was then purified by CsCl centrifugation. The chromosomal DNA from T. reesei was partially digested with MboI and sized by sucrose density gradient centrifugation. Fifteen-20 kb fragments were ligated to Bam HI-cleaved λ 1050 DNA. In vitro packaging of the

recombinant molecules was carried out using packaging extracts prepared by the method of Hohn as described by Maniatis et al. (25).

Recombinant phages were transferred from the agar to nitro-cellulose filters (Schleicher & Schüll, BA 85) as described by Benton and Davis (34). cDNAs made from induced mRNA (described earlier) and from mRNA isolated from fungus grown in the presence of glucose were used as probes. cDNA first strand synthesis was carried out by the procedure of Efstradiatis et al. (35) but using 10 μ Ci of 32 p α ATP per 50 μ l reaction. The in situ plaque hybridization was carried out according to Maniatis et al. (25). Hybridization was detected by autoradiography of the filters on Kodak X-OMAT film. Positive plaques were picked into 1 ml of SM (25) and a drop of chloroform and stored at -4 C.

Hybrid phage hybridizing only to cDNA made with induced mRNA containing cellulase coding sequences were purified extensively and retested by hybridization to both probes. A number of different hybrid clones that hybridized strongly to the induced cellulase probe were identified and selected for further analysis.

The hybrid phages containing genes induced when the fungus produces cellulases were first grouped according to their restriction enzyme patterns. Then the particular cellulase gene in each group was identified by hybrid selection of messenger RNA.

DBM paper was obtained from Schleicher and Schüll (Keene, NH) and activated according to the maker's instructions. Binding of DNA to the activated paper and RNA hybridization and elution was carried out according to Maniatis et al. (25). RNA was translated with a rabbit reticulocyte lysate supplied by Amersham International Ltd. and the proteins produced were labeled with ³⁵S-methionine. The proteins were analysed by autoradiography on Kodak X-OMAT film after separation on a 7-15 % polyacrylamide

gradient denaturing gel.

The size of the proteins obtained from particular phage by hybrid selection and their cross reaction with specific antiserum is shown in Table 2.

Table 2.

Hybrid Phage No.	44A	W17A	W12A
Mol. weight of major protein produced from hybrid selected message	67 000	48 000	53 000
Cross reaction of major protein with antisera against			
CBH I	+	-	-
CBH II	-	+	-
ENDO II	-	-	+

Single and double digests of the clone 44A, were analyzed on 0.6 % and 1.5 % agarose gels. The fragments were electrophoretically transferred to Gene Screen membranes (New England Nuclear, MA) and hybridized to the induced cDNA probe as instructed by the manufacturer.

This procedure permitted the construction of restriction enzyme maps of the three cellulose genes. These restriction enzyme maps are shown in figures 1, 2 and 3.

The nucleotide sequence of the CBH I, CBH II and ENDO II genes was generated by dideoxy sequencing (36) using restriction enzyme fragments or DNA fragments obtained by the "shotgun" procedure (37).

The construction of a yeast vector containing the CBH I chromosomal gene

The hybrid phage 44A (11) DNA containing the CBH I of Trichoderma reesei hypercellulolytic mutant strain VTT-D-80133, was digested with Pst I to give a mixture of fragments one of which being about 12 kb and containing the entire CBH I gene with its own regulatory sequences. The resulting DNA-fragments were ligated with the yeast cosmid p3030 digested partially with the same enzyme.

The yeast strain OLi was transformed to his⁺ with the DNA-mixture described above. Transformation was carried out essentially as described by Gerbaud et al. (38). Transformed cells were plated on yeast minimal medium with leucin and uracil but lacking histidine..

The clones were further tested in situ plaque hybridization for the presence of the CBH I gene originated from T. reesei.

The presence of an intact CBH I gene in yeast was ensured by isolating total DNA (39) from a transformant colony and digesting it with restriction enzymes Bgl II and Hinc II. DNA was transferred to nitrocellulose filter (40) from agarose gel and hybridized to a M13 probe (41) containing the 0.7 kb Eco RI fragment from CBH I gene. Figure 4 shows the construction of a hybrid plasmid containing the CBH I gene.

Isolation of full length cDNAs coding for the enzymes CBH I, CBH II and ENDO II

A cDNA bank from T. reesei was made from induced mRNA isolated from cells as described earlier. However, after the frozen mycelia had been ground under liquid nitrogen it was suspended in 5 volumes a guanidinium isothiocyanate buffer as described by Maniatis et al. (25). The RNA preparation was then carried out as described (42).

cDNA first strand synthesis was carried out according to Maniatis (25) and the second strand was carried out according to Gubler and Hoffmann (43). The double stranded cDNA was then treated with T_4 -polymerase to give blunt ends and small cDNAs less than 500 nucleotides long removed by passage through a CL-4B column (Pharmacia). Long cDNAs were then ligated to a Sma I digested and phosphatase treated preparation of pUC 8 vector. The ligation mixture was used to transform E. coli strain JM 105 and the cDNA bank was stored on nitrocellulose filters.

Full length cDNAs coding for CBH I, CBH II and ENDO II were isolated from a cDNA bank using specific restriction fragments as probes. For the identification of CBH I, a radioactive Eco RI-Hind III fragment from the 5' end of the chromosomal gene was used to identify long cDNAs. A plasmid pTT01 from a clone containing sequences homologous to this Eco RI-Hind III fragment was further characterized by sequencing of the cDNA ends by double stranded dideoxy sequencing. 1 μ g of purified plasmid was denatured in 0.4 M NaOH at room temperature for 5 minutes at a concentration of 100 ng/ μ l. 5 μ l of sequencing or reverse sequencing primer (Amersham) was added and the mixture was precipitated with ethanol. After washing the pellet was resuspended in 10 μ l at 14 mM Tris pH 8 - 7 mM MgCl₂. Sequencing reactions were done according to general methods (36) except that temperature was kept at 37°C. CBH II cDNAs were isolated using a Pvu II fragment from the 5' end of the chromosomal gene and the plasmid pTT09 characterized as for the CBH I cDNA. ENDO II cDNAs were identified using a Kpn I-Sal I fragment from the 5' end of the gene and plasmid pTT11 also characterized as for the CBH I cDNA. All cDNAs were then sequenced to determine that their sequence corresponded to that of the gene from which they are transcribed. The DNA sequences of CBH II and ENDO II cDNAs are shown in Figures 5 and 6. The cDNA sequence of CBH I was identical to that already described (10).

The construction of expression vectors containing cDNAs for the production of fungal cellulases in yeast

The efficient yeast expression vector pMA 91 has been assembled using the regulatory sequences of the yeast phosphoglycerokinase (PGK) gene (23). The sequences coding for the amino acid sequence of the enzyme have been removed from the gene and replaced by a single Bgl II site. This deleted gene has then been inserted into a yeast/coli shuttle plasmid.

a) CBH I expression vector (Figure 7)

The CBH I cDNA was removed from plasmid (pTT01 Figure 7) by digestion with Hinc II and the cDNA fragment isolated from an agarose gel.

pMA 91, the expression vector was cleaved with Bgl II and the ends were filled in with the Klenow fragment. The vector was treated with phosphatase, ligated to the cDNA and transformed into E. coli strain HB101 by selection for expression of the vector leucine gene (Figure 7). Plasmid DNA was isolated from a number of transformants and those clones containing the cDNA insert in the correct orientation with respect to the PGK promoter - as identified by restriction enzyme analysis - were retained. DNA from one of these clones (pMP 11) was then transformed into yeast strain MT 302-1c by the method described earlier by selection of the leucine marker of pMA 91 resulting in strain VTT-RC-84011.

b) CBH II expression vector (Figure 8)

CBH II cDNA was removed from plasmid pTT 09 using Eco RI and Bam HI. The ends of the DNA were filled in with Klenow fragment. The cDNA fragment was then isolated from an agarose gel and ligated to the vector pMA 91 prepared as for CBH I.

The ligation mix was transferred into HB101 and clones containing the cDNA in the correct orientation identified. Figure 8 shows

the DNA sequence at the junctions between pMA 91 and the cDNA.

Plasmid pMP 29 with the cDNA in the correct orientation was then used to transform yeast MT302-1c by selection for the leucine marker to give strain VTT-RC-84012.

c) ENDO II expression vector (Figure 9)

The ENDO II cDNA was transferred to pMA 91 exactly in the same way as CBH II cDNA. Figure 9 shows the DNA sequences at the junctions between pMA 91 and the ENDO II cDNA. The plasmid, pMP 311 containing the ENDO II cDNA in the correct orientation was transferred to yeast as described earlier to give strain VTT-RC-84013.

Culturing the hybrid yeast strains to produce the cellulolytic enzymes, CBH I, CBH II and ENDO II

Strain VTT-RC-84001 containing YEpNP03 was grown in a yeast minimal medium with leucine and uracil for three days after which complete medium (1/3 volume) was then added to allow the cells to pass through one more division.

Strains VTT-RC-84011 (CBH I cDNA), VTT-RC-84012 (CBH II cDNA) and VTT-RC-84013 (ENDO II cDNA) were grown in a yeast minimal medium containing arginine, histidine and adenine for three days after which complete medium 1/3 volume was added to allow the cells to pass through one more division. The final volume of the cultures was about 150 ml.

Preparation of different fractions for analysis of the location of enzyme activity

Three fractions were prepared from hybrid yeast cultures for analysis of enzyme activity. Fraction 1 comprised the growth medium without the cells. Fraction 2 comprises the supernatant left when protoplasts are pelleted and fraction 3 comprises the

supernatant of lysed protoplasts.

After cultivation yeast cells were collected by centrifugation and the supernatant was saved (Fraction 1). The resulting pellet was washed twice with distilled water and 1.2 M sorbitol. The pellet was then resuspended in protoplasting buffer (1.2 M sorbitol, 10 mM Tris and 10 mM CaCl₂, pH 7.6) and Zymolyase 60000 was added at a concentration of 30 µg/ml of protoplasting suspension. Suspension was incubated in a waterbath at 37 °C for 60 minutes with gentle shaking. The protoplasts so formed were pelleted and the resulting supernatant (periplasmic cell contents) (Fraction 2) saved for enzyme activity determinations. In some cases fractions 1 and 2 were concentrated by ultrafiltration (Amicon). Protoplast pellets were washed with cold 1.2 M sorbitol and resuspended in 1.2 ml of 5 mM citrate buffer pH 5.0, pelleted and the supernatant was saved (Fraction 3).

Measurement of cellulase enzyme activity produced by the hybrid yeasts

1 CBH I activity from VTT-RC-84001

The three different fractions were tested for CBH I enzyme activity using amorphous ball milled cellulose which is attached only by cellobiohydrolases (44). The total protein concentration of the samples was about 300 µg/ml. Hydrolysis of the substrate caused by active cellobiohydrolase enzyme was measured by following the change in absorbance at 620 nm. CBH I type activity was found only in fraction 2, the periplasmic or intramural space.

Figure 10 shows the activity of the CBH I enzyme produced by the yeast strain VTT-RC-84001 and secreted into the intramural space as compared to the control yeast containing only vector p3030 DNA and 1 µg of Trichoderma CBH I. This Figure shows that the hybrid yeast strain produces active CBH I which appears to be

at least as resistant to incubation at 50°C for 3 days as in the native enzyme. The CBH I produced by the yeast represents 1-2 % of the protein of intramural space protein.

As the intron sequences of fungus are different from those of yeast it is not likely that yeast would process off the fungus gene introns. Probably for that reason the product of the chromosomal gene remains in the yeast periplasmic space and is not secreted from the cell as is the product coded by the cDNA sequence. This result suggests that transferring the chromosomal gene coding for CBH I to yeast, results in the production of a smaller protein, which, however, has the same type of activity as the full length cellulase.

2 CBH I activity from VTT-RC-84011

The three different fractions were tested for CBH I enzyme activity as just described. However, in this case, most of the CBH I type activity was found in the growth medium. The results, with a final protein concentration during hydrolysis of 5 µg/ml is very similar to that shown in Figure 10. The CBH I enzyme produced with this construction represented 1-5 % of total cell protein.

3 CBH II activity from VTT-RC-84012

The three different fractions were tested from cellobiohydrolase activity as described for strain VTT-RC-84001. As with strain VTT-RC-84011, most of the cellobiohydrolase type activity was found in the growth medium. The results with a final protein concentration of 10 µg/ml is similar to that shown in Figure 10. The CBH II enzyme produced with this construction represented 1-5 % of total cell protein.

4 ENDO II activity from VTT-RC-84013

The three different fractions were tested for endoglucanase activity by following the hydrolysis of 0.1 % β -glucan at 50 °C.

The reducing sugars liberated in 5 minutes (overnight) were measured as glucose using the dinitro salisylic acid method (45). Most of the ENDO II activity was found secreted into the growth medium. The ENDO II enzyme produced with this construction represented 1-5 % of total cell protein.

It is considered that the invention and many of its attendant advantages will be understood from the foregoing description and that it will be apparent that various changes may be made in the steps of the described method for mature protein synthesis without departing from the spirit and scope of the invention or sacrificing all of its material advantages, the method herein before described being merely a preferred embodiment.

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WHAT IS CLAIMED IS:

1. A DNA sequence which codes for a fungal cellulase enzyme, or its single or multiple base substitutions, deletions, insertions, or inversions, which is derived from natural, synthetic or semi-synthetic sources and which is capable, when correctly combined with an expression vector, of expressing a non-native protein having cellulolytic activity upon transformation of a host organism by the vector, said DNA sequence coding for substantially the following amino acid sequence:

MetIleValGlyIleLeuThrThrLeuAlaThrLeuAlaThrLeuAlaAlaSerValProLeuGluGluArgGlnAlaCysSerSerVal
 TrpGlyGlnCysGlyGlyGlnAsnTrpSerGlyProThrCysCysAlaSerGlySerThrCysValTyrSerAsnAspTyrTyrSerGln
 CysLeuProGlyAlaAlaSerSerSerSerSerThrArgAlaAlaSerThrThrSerArgValSerProThrThrSerArgSerSerSer
 AlaThrProProGlySerThrThrThrArgValProProValGlySerGlyThrAlaThrTyrSerGlyAsnProPheValGlyVal
 ThrProTrpAlaAsnAlaTyrTyrAlaSerGluValSerSerLeuAlaIleProSerLeuThrGlyAlaMetAlaThrAlaAlaAlaAla
 ValAlaLysValProSerPheMetTrpLeuAspThrLeuAspLysThrProLeuMetGluGlnThrLeuAlaAspIleArgThrAlaAsn
 LysAsnGlyGlyAsnTyrAlaGlyGlnPheValValTyrAspLeuProAspAspCysAlaAlaLeuAlaSerAsnGlyGluTyrSer
 IleAlaAspGlyGlyValAlaLysTyrLysAsnTyrIleAspThrIleArgGlnIleValValGluTyrSerAspIleArgThrLeuLeu
 ValIleGluProAspSerLeuAlaAsnLeuValThrAsnLeuGlyThrProLysCysAlaAsnAlaGlnSerAlaTyrLeuGluCysIle
 AsnTyrAlaValThrGlnLeuAsnLeuProAsnValAlaMetTyrLeuAspAlaGlyHisAlaGlyTrpLeuGlyTrpProAlaAsnGln
 AspProAlaAlaGlnLeuPheAlaAsnValTyrLysAsnAlaSerSerProArgAlaLeuArgGlyLeuAlaThrAsnValAlaAsnTyr
 AsnGlyTrpAsnIleThrSerProProSerTyrThrGlnGlyAsnAlaValTyrAsnGluLysLeuTyrIleHisAlaIleGlyProLeu
 LeuAlaAsnHisGlyTrpSerAsnAlaPhePheIleThrAspGlnGlyArgSerGlyLysGlnProThrGlyGlnGlnGlnTrpGlyAsp
 TrpCysAsnValIleGlyThrGlyPheGlyIleArgProSerAlaAsnThrGlyAspSerLeuLeuAspSerPheValTrpValLysPro
 GlyGlyGluCysAspGlyThrSerAspSerSerAlaProArgPheAspSerHisCysAlaLeuProAspAlaLeuGlnProAlaProGln
 AlaGlyAlaTrpPheGlnAlaTyrPheValGlnLeuLeuThrAsnAlaAsnProSerPheLeu

or a portion thereof.

2. A DNA sequence according to Claim 1 which codes for the cellobiohydrolase II activity and has substantially the following nucleotide sequence:

CTTGTAAAGATCACCTCTGTGATTGCACC

ATGATTGTCGGCATTCTCACCACGCTGGCTACGCTGGCCACACTCGCAGCTAGTGTGCCTCTAGAGGAGCGGCAAGCTTGETCAAGCGTC
 TGGGGCCAATGTGGTGGCCAGAATTGGTGGGTCCGACTTGCTGTGCTTCGGAAGCAGCATGCGTCTACTCCAACGACTATTACTCCCAG
 TGTCTTCCGGCGCTGCAAGCTCAAGCTCGTCCACGCGCGCGCTCGAAGCTTCTCGAGTATCCCCACAACATCCCGGTGAGCTCC
 GCGACGCCTCCACCTGTTCTACTACTACCAGAGTACCTCCAGTCGGATCGGGAACCGCTACGTATTGAGCAACCTTTTGTGGGGTC
 ACTCCTTGGGCCAATGCATATTACGCCTCTGAAGTTAGCAGCCTCGCTATTCCTAGCTTGACTGGAGCCATGGCCACTGCTGCAGCAGCT
 GTCGCAAAGGTTCCCTCTTTATGTGGCTAGATACTCTTGACAAGACCCCTCTCATGGAGCAAACCTTGGCCGACATCCGACCGCCAAAC
 AAGAATGGCGGTAATATGCCGGACAGTTTGTGGTGTATGACTTGCCGGATCGCGATTGCGCTGCCCTTGCTCGAATGGCAATACTCT
 ATTGCCGATGGTGGCGTCGCCAAATATAAGAACTATATCGACACCATTGCTCAAATTGTGCGTGAATATTCGATATCCGGACCCCTCTG
 GTTATTGAGCCTGACTCTCTTGCCAACTGGTGACCAACCTCGGTACTCCAAAGTGTGCAATGCTCAGTCAGCCTACCTTGAGTGCATC
 AACTACGCCGTACACAGCTGAACCTTCAAATGTTGCGATGTATTGGACGCTGGCCATGAGGATGGCTTGGCTGGCCGGCAAACCAA
 GACCGGCCCGCTCAGCTATTTGCAAATGTTTACAAGAATGCATCGTCTCGAGAGCTCTTCGCGGATTGGCAACCAATGTGCGCAACTAC
 AACGGGTGGAACATTACCAGCCCCCATCGTACACGCAAGGCAACGCTGTCTACAACGAGAAGCTGTACATCCAGCTATTGGACCTCTT
 CTTGCCAATCACGGCTGGTCCAACGCCTTCTTCATCACTGATCAAGGTCATCGGGAAAGCAGCCTACCGGACAGCAACAGTGGGGAGAC
 TGGTGCAATGTGATCGGCACCGGATTGGTATTGCGCCATCCGCAAACTGGGGACTCGTTGCTGGATTGTTTGTCTGGGTCAAGCCA
 GCGCGCGAGTGTGACGGCACCAGCGACAGCAGTGGCCACGATTTGACTCCCACTGTGCGCTCCAGATGCCCTTGCAACCGCGCCTCAA
 GCTGGTGTCTGGTTCAGGCTACTTTGTGCAGCTTCTCACAACGCAAAACCATCGTTCCTGTAAAGGCTTTCGTGACCGGGCTTCAAAC
 AATGATGTGCGATGGTGTGGTCCCGGTCGCGGAGTCTTTGTCTACTTTGGTTGTCTGTCGAGGTGGTAGACCGCAAATGAGCAACT
 GATGGATTGTTGCCAGCGATACTATAATTCACATGGATGGTCTTTGTGATCAGTAGGCTAGAGAGAGAGAGAACATCTATCCACAAT
 GTGAGTGTCTATT

or a portion thereof.

3. A DNA sequence according to Claim 1 wherein the DNA is cDNA copy of mRNA coding for cellobiohydrolase II in Trichoderma reesei.

4. A signal sequence responsible of the secretion of a proteinaceous material extracellularly, said signal sequence having substantially the following amino acid sequence:

MetIleValGlyIleLeuThrThrLeuAlaThrLeuAlaThrLeuAlaAlaSerValProLeuGluGluArg

5. A DNA sequence which codes for a fungal cellulase enzyme, or its single or multiple base substitutions, deletions, insertions, or inversions, which is derived from natural, synthetic or semi-synthetic sources and which is capable, when correctly combined with an expression vector, of expressing a non-native protein having cellulolytic activity upon transformation of a host organism by the vector, said DNA sequence coding for substantially the following amino acid sequence:

MetAlaProSerValThrLeuProLeuThrThrAlaIleLeuAlaIleAlaArgLeuValAlaAlaGlnGlnProGlyThrSerThrPro
 GluValHisProLysLeuThrThrTyrLysCysThrLysSerGlyGlyCysValAlaGlnAspThrSerValValLeuAspTrpAsnTyr
 ArgTrpMethisAspAlaAsnTyrAsnSerCysThrValAsnGlyGlyValAsnThrThrLeuCysProAspGluAlaThrCysGlyLys
 AsnCysPheIleGlnGlyValAspTyrAlaAlaSerGlyValThrThrSerGlySerSerLeuThrMetAsnGlnTyrMetProSerSer
 SerGlyGlyTyrSerSerValSerProArgLeuTyrLeuLeuAspSerAspGlyGluTyrValMetLeuLysLeuAsnGlyGlnGluLeu
 SerPheAspValAspLeuSerAlaLeuProCysGlyGluAsnGlySerLeuTyrLeuSerGlnMetAspGluAsnGlyGlyAlaAsnGln
 TyrAsnThrAlaGlyAlaAsnTyrGlySerGlyTyrCysAspAlaGlnCysProValGlnThrTrpArgAsnGlyThrLeuAsnThrSer
 HisGlnGlyGlnGlyPheCysCysAsnGluMetAspIleLeuGluGlyAsnSerArgAlaAsnAlaLeuThrProHisSerCysThrAla
 ThrAlaCysAspSerAlaGlyCysGlyPheAsnProTyrGlySerGlyTyrLysSerTyrTyrGlyProGlyAspThrValAspThrSer
 LysThrPheThrIleIleThrGlnPheAsnThrAspAsnGlySerProSerGlyAsnLeuValSerIleThrArgLysTyrGln.....
 ValAspIleProSerAlaGlnProGlyGlyAspThrIleSerSerCysProSerAlaSerAlaTyrGlyGlyLeuAlaThrMetGlyLys
 AlaLeuSerSerGlyMetValLeuValPheSerIleTrpAsnAspAsnSerGlnTyrMetAsnTrhLeuAspSerGlyAsnAlaGlyPro
 CysSerSerThrGluGlyAsnProSerAsnIleLeuAlaAsnAsnProAsnThrHisValValPheSerAsnIleArgTrpGlyAspIle
 GlySerThrThrAsnSerThr.....SerSerThrThrSerSerSerProSerCysThrGlnThrHisTrpGlyGlnCys
 GlyGlyIleGlyTyrSerGlyCysLysThrCysThrSerGlyThrThrCysGlnTyrSerAsnAspTyrTyrSerGlnCysLeuxxx

or a portion thereof.

6. A DNA sequence according to Claim 5, which codes for the endoglucanase II activity and has substantially the following nucleotide sequence:

CCCCCTATCTTAGTCCTTCTTGTGTCCAAA
 ATGGCGCCCTCAGTTACACTGCCGTTGACCACGGCCATCCTGGCNATTGCCCGGCTCGTCGCCGCCAGCAACCGGGTACAGCACCCCC
 GAGGTCCATCCCAAGTTGACAACCTACAAGTGACAAAGTCGGGGGGTGGTGCGCCAGGACACCTCGGTGGTCTTGAAGTGAAGTAC
 CGCTGGATGCACGACGAACTACAACCTCGTGACCGCTCACCGCGCGCTCAACACCACGCTCTGCCCTGACGAGGCGACCTGTGGCAAG
 AACTGCTTATCCAGGGCTCGACTACGCCGCTCGGGCGTCACGACCTCGGGCAGCAGCCTACCATGAACCAGTACATGCCCAGCAGC
 TCTGGCGGTACAGCAGGCTCTCTCTCGGCTGTATCTCTGGACTCTGACGGTGAGTACGTGATGCTGAAGCTCAACGCCAGGAGCTG
 AGCTTCGACCTCGACTCTCTGTCTGCGGTGGAGAGAAGCGCTCGCTCTACCTGTCTCAGATGGACGAGAACGGGGGCGCAACCAG
 TATAACACGGCCGGTGCCAACTACGGGAGCGGCTACTGCGATGCTCAGTGCCCCGTCCAGACATGGAGGAACGGCACCCCTCAACACTAGC
 CACGAGGCCAGGGCTTCTGCTGCAACGAGATGGATATCCTGGAGGGCAACTCGAGGGCGAATGCCTTGACCCCTCACTCTTGACCGGCC
 ACGGCCTGCGACTCTGCGGTTGCGGCTTCAACCCCTATGGCAGCGGCTACAAAAGCTACTACGGCCCGGAGATACGTTGACACCTCC
 AACACCTTCACCATCATCACCAGTTCAACACGGACAACGGCTCGCCCTCGGGCAACCTTGAGCATCACCCGCAAGTACCAGXXXXX
 GTGCAGATCCCCAGCGCCAGCCCGGGCGGACACCATCTGCTCTGCGGCTCGGCTCAGCCTACGGCGGCTCGCCACCATGGGCAAG
 GCCCTGAGCAGCGCATGGTCTCGTSTTCAGCATTTGGAACGACAACAGCCAGTACATGAAGTGGCTCGACAGCGCAACGCGGCCCC
 TGCAGCAGCACCGAGGGCAACCCATCCAACATCCTGGCCAACAACCCCAACACGACGCTGCTCTCTCCAACATCCGCTGGGGAGACATT
 GGGTCTACTACGAATCGACT....x.....x..GAGCTCGAGACTTCGAGCAGCCGAGCTGCACGCACTCACTGGGGCAGTGC
 GGTGGCATTGGGTACAGCGGTGCAAGACGTGCACGTGGGCACTACGTGCCAGTATAGCAACGACTACTACTCGCAATGCCTTTAGAGC
 GTTGACTTGCTCTGGTCTGTCCAGACGGGGGACGATAGAATGCGGGCACGAGGGAGCTCGTAGACATTGGGCTTAATATAAGACA
 TGCTATGTTGTATCTACATTAGCAATGACAACAAATGAAAAAGAACTTATCAAGC

or a portion thereof.

7. A DNA sequence according to Claim 5, wherein the DNA is cDNA copy of mRNA coding for endoglucanase II in Trichoderma reesei.

8. A signal sequence responsible of the secretion of a proteinaceous material extracellularly, said signal sequence having substantially the following amino acid sequence:

MetAlaProSerValThrLeuProLeuThrThrAlaIleLeuAlaIleAlaArgLeuValAlaAlaGln

9. A recombinant DNA vector comprising a DNA sequence according to any of Claims 1-3 or any of Claims 5-7 or a signal sequence according to Claim 4 or 8, said vector being able to replicate and express in a suitable host organism.

10. A recombinant DNA vector according to Claim 9 wherein the DNA sequence of Claims 1-3 is ligated to plasmid pMA 91.

11. A recombinant DNA vector according to Claim 10 said vector being pMP 29.

12. A recombinant DNA vector according to Claim 9 wherein the DNA sequence of Claims 5-7 is ligated to plasmid pMA 91.

13. A recombinant DNA vector according to Claim 12 said vector being pMP 311.

14. A recombinant DNA vector according to any of Claims 9-13, which is able to replicate and express in yeast.

15. A recombinant DNA vector according to any of Claims 9-14, which is able to replicate and express in genus Saccharomyces.

16. A recombinant DNA vector comprising the following combination:

chromosomal gene isolated from Trichoderma reesei coding for cellobiohydrolase I ligated to plasmid p3030, or cDNA copy of mRNA isolated from T. reesei coding for cellobiohydrolase I ligated to plasmid pMA 91, said vector being able to replicate and express in yeast.

17. A recombinant DNA vector according to Claim 16, wherein the chromosomal gene coding for cellobiohydrolase I is ligated to the restriction enzyme cleavage site of partially digested Pst I of plasmid p3030 and wherein the vector is YE pNP03.

18. A recombinant DNA vector according to Claim 16, wherein the cDNA sequence coding for cellobiohydrolase I is ligated to the restriction enzyme cleavage site of Bgl II of plasmid pMA 91 and is under the control of the regulatory regions of phosphoglycerokinase gene, and wherein the vector is pMP 11.
19. A recombinant DNA vector according to Claim 16, said vector being able to replicate and express in genus Saccharomyces.
20. A yeast strain containing one or several of the following DNA sequences:
a DNA sequence according to any of Claims 1-3 or a DNA sequence according to any of Claims 5-7 or a signal sequence according to Claim 4 or 8 or a chromosomal gene or cDNA sequence coding for cellobiohydrolase I.
21. A yeast strain according to Claim 20, which comprises a recombinant DNA vector containing the DNA sequence according to any of Claims 1-3.
22. A yeast strain according to Claim 21, wherein the DNA sequence of Claims 1-3 is ligated to plasmid pMA 91 and said recombinant DNA vector being pMP 29.
23. A yeast strain according to Claim 20, which comprises a recombinant DNA vector containing the DNA sequence according to any of Claims 5-7.
24. A yeast strain according to Claim 23, wherein the DNA sequence of Claims 5-7 is ligated to plasmid pMA 91 and said recombinant DNA vector being pMP 311.
25. A yeast strain according to Claim 20, which contains the recombinant DNA vector YE pNP03, wherein the chromosomal gene isolated from T. reesei and coding for cellobiohydrolase I is ligated to plasmid p3030.

26. A yeast strain according to Claim 20, which contains the recombinant DNA vector pMP 11, wherein the cDNA sequence isolated from T. reesei and coding for cellobiohydrolase I is ligated to plasmid pMA 91.

27. A yeast strain according to any of Claims 20-26, said yeast strain being a member of genus Saccharomyces.

28. A yeast strain according to any of Claims 20-27, said yeast strain comprising:

S. cerevisiae VTT-RC-84001 (NCYC R 128)

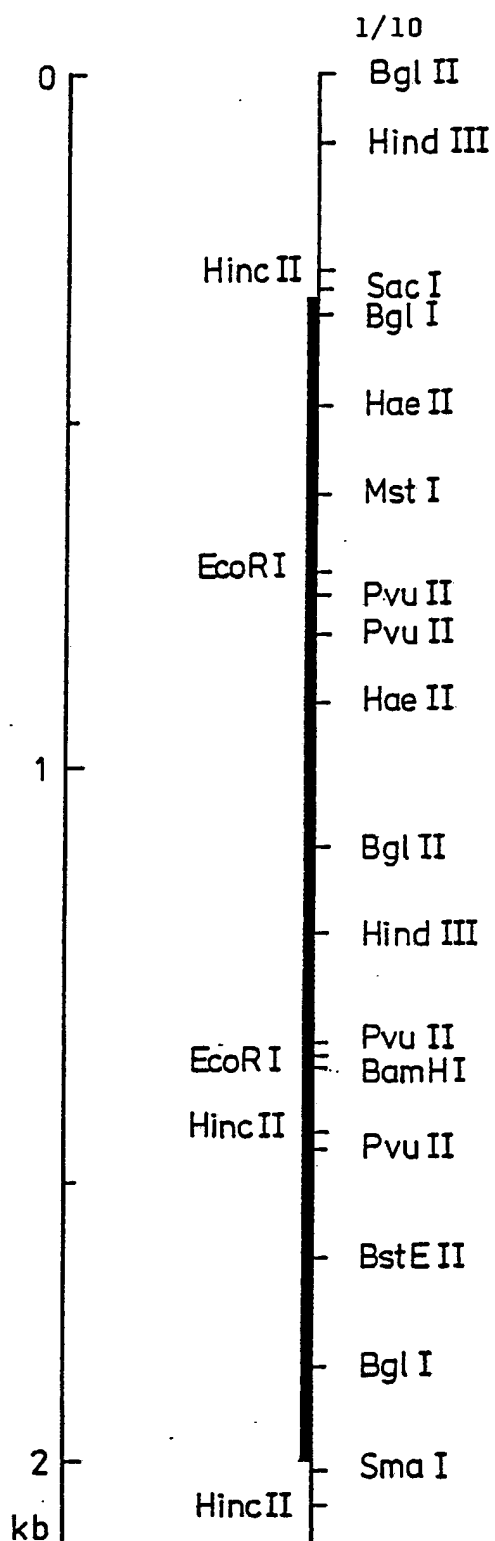
S. cerevisiae VTT-RC-84011

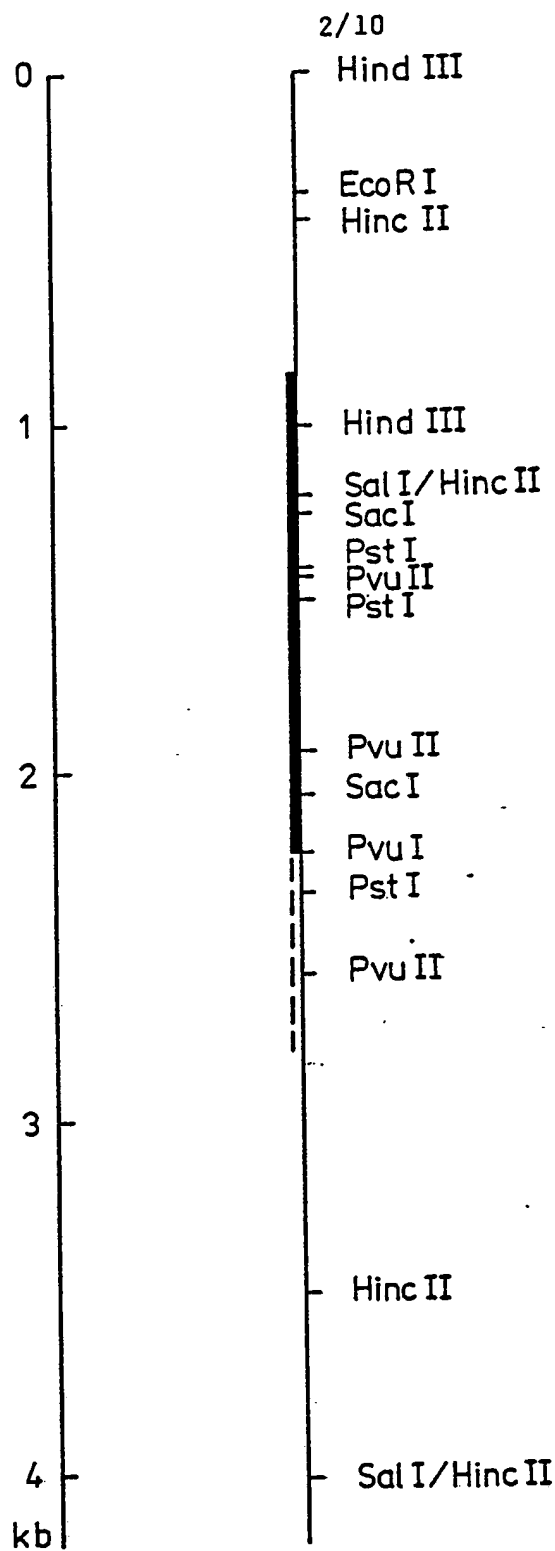
S. cerevisiae VTT-RC-84012

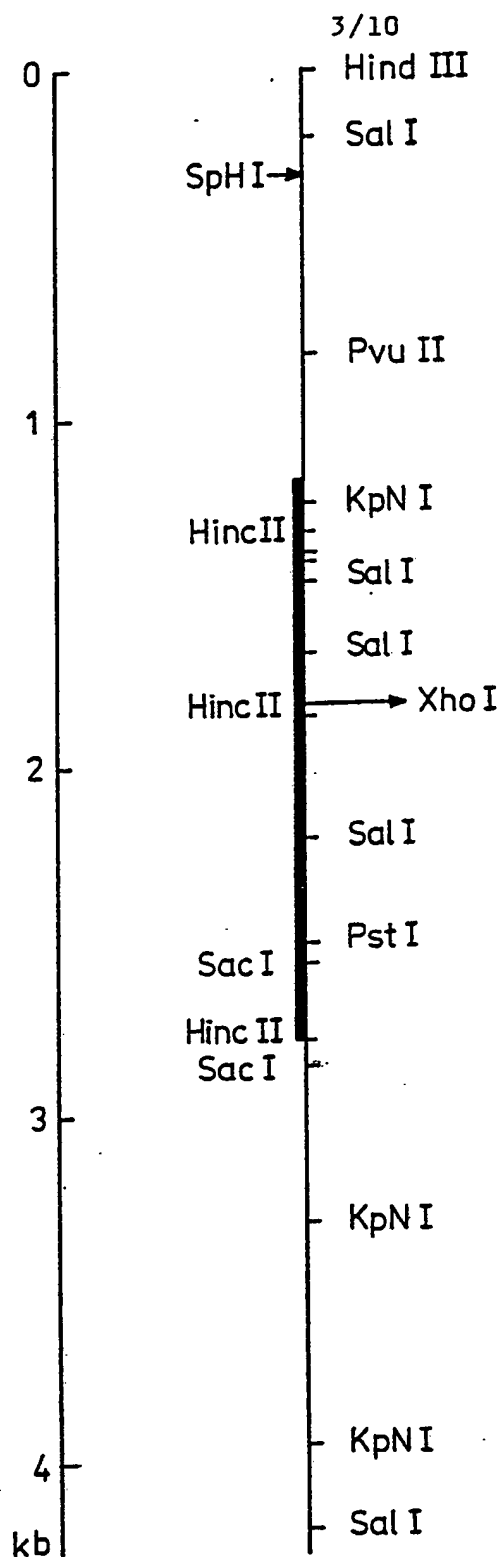
S. cerevisiae VTT-RC-84013.

29. A method for constructing a recombinant DNA vector according to Claim 9 or 16, said method comprising ligating a DNA sequence according to any of Claims 1-3 or 5-7 or a signal sequence according to Claim 4 or 8 to a plasmid able to replicate and express in yeast or ligating a chromosomal gene coding for cellobiohydrolase I to plasmid p3030 or a cDNA sequence coding for cellobiohydrolase I to plasmid pMA 91.

30. A method for constructing a yeast strain according to Claim 20, said method comprising transforming a yeast strain by one or several of the recombinant DNA vectors of Claims 9-19.

*Fig. 1*

*Fig. 2*

*Fig.3*

4/10

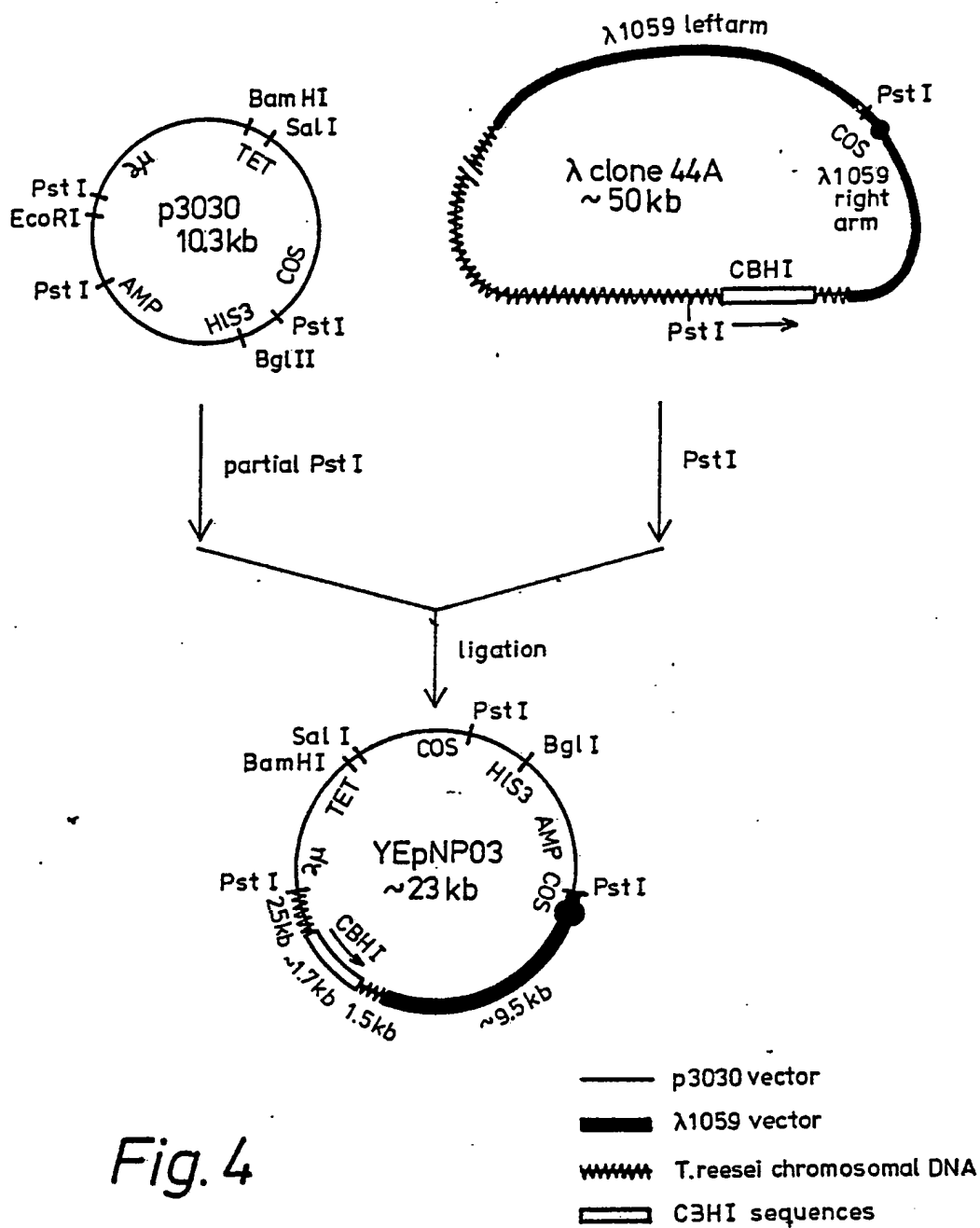


Fig. 4

5/10

EcoRI CBH II CNA
 ACCATGATTACGAATTCCTTGTAAAGTACCCCTCTGTGATTGCACC
 pUC8

— CBH II signal sequence — Hind III
 ATGATTGTCGGCATTCTCACCACGCTGGCTACGCTGGCCACACTCGCAGCTAGTGTGCCTCTAGAGGAGCGGCAAGCTTGTCTCAAGCGTC
 MetIleValGlyIleLeuThrThrLeuAlaThrLeuAlaThrLeuAlaAlaSerValProLeuGluGluArgGlnAlaCys. SerSerVal
 CBHII mature protein

Acc I
 TGGGGCCAATGTGGTGGCCAGAATTGGTGGGTCCGACTTGTGTCTTCGGAAGCACATGCGTCTACTCCAACGACTATTACTCCAG
 TrpGlyGlnCysGlyGlyGlnAsnTrpSerGlyProThrCysCysAlaSerGlySerThrCysValTyrSerAsnAspTyrTyrSerGln

Acc I Sac I
 TGTCTTCCGGCGCTGCAAGCTCAAGCTCGTCCACGCGCGCGTGGACGACTTCTCGAGTATCCCCACAACATCCCGGTGAGCTCC
 CysLeuProGlyAlaAlaSerSerSerSerThrArgAlaAlaSerThrThrSerArgValSerProThrThrSerArgSerSerSer

GCGACGCTCCACCTGGTTCTACTACTACCAGAGTACCTCCAGTCGGATCGGGAACCGCTACGTATTCAGGCAACCCCTTTTGTGGGGTC
 AlaThrProProProGlySerThrThrThrArgValProProValGlySerGlyThrAlaThrTyrSerGlyAsnProPheValGlyVal

Pst I Pvu II
 ACTCCTTGGGCAATGCATATTACGCCTCTGAAGTTAGCAGCCTCGCTATTCTAGCTTGACTGGAGCCATGGCCACTGCTGCAGCAGT
 ThrProTrpAlaAsnAlaTyrTyrAlaSerGluValSerSerLeuAlaIleProSerLeuThrGlyAlaMetAlaThrAlaAlaAlaAla

GTCCGAAAGGTTCCCTCTTTATGTGGCTAGATACTCTTGACAAGACCCCTCTCATGGAGCAAACCTTGGCCGACATCCGACCGCCAAAC
 ValAlaLysValProSerPheMetTrpLeuAspThrLeuAspLysThrProLeuMetGluGlnThrLeuAlaAspIleArgThrAlaAsn

AAGAATGGCGGTAACATATGCGGACAGTTTGTGGTGTATGACTTGCGGGATCGCGATTGCGCTGCCCTTGCCTCGAATGGCGAATACTCT
 LysAsnGlyGlyAsnTyrAlaGlyGlnPheValValTyrAspLeuProAspArgAspCysAlaAlaLeuAlaSerAsnGlyGlyTyrSer

ATTGCCGATGGTGGCTCGCCAAATATAAGAACTATATCGACACCATTCGTCAAATTGTCGTGGAATATTCGGATATCCGACCCCTCTG
 IleAlaAspGlyGlyValAlaLysTyrLysAsnTyrIleAspThrIleArgGlnIleValValGluTyrSerAspIleArgThrLeuLeu

GTTATTGAGCTGACTCTCTTGCCAACTGGTGACCAACCTCGTACTCCAAAGTGTGCAATGCTCAGTCAGCCTACCTTGAGTGCATC
 ValIleGluProAspSerLeuAlaAsnLeuValThrAsnLeuGlyThrProLysCysAlaAsnAlaGlnSerAlaTyrLeuGluCysIle

Pvu II
 AACTACGCGCTCACACAGCTGAACCTTCCAAATGTTGCGATGTATTTGACGCTGGCCATGCAGGATGGCTTGGTGGCCGGCAACCAA
 AsnTyrAlaValThrGlnLeuAsnLeuProAsnValAlaMetTyrLeuAspAlaGlyHisAlaGlyTrpLeuGlyTrpProAlaAsnGln

Sac I
 GACCCGGCGCTCAGCTATTTGCAATGTTTACAAGATGCATCGTCTCCGAGAGCTCTTCGCGGATTGGCAACCAATGTCCGCAACTAC
 AspProAlaAlaGlnLeuPheAlaAsnValTyrLysAsnAlaSerSerProArgAlaLeuArgGlyLeuAlaThrAsnValAlaAsnTyr

Acc I
 AACGGGTGGAACATTACAGCCCCCATCGTACACGCAAGGCAACGCTGTCTACAACGAGAAGCTGTACATCCAGCTATTGGACCTCTT
 AsnGlyTrpAsnIleThrSerProProSerTyrThrGlnGlyAsnAlaValTyrAsnGluLysLeuTyrIleHisAlaIleGlyProLeu

Pvu I
 CTTGCCAATCACGGCTGGTCCAAACGCTTCTTCATCACTGATCAAGGTCGATCGGAAAGCAGCCTACCGGACAGCAACAGTGGGGAGAC
 LeuAlaAsnHisGlyTrpSerAsnAlaPhePheIleThrAspGlnGlyArgSerGlyLysGlnProThrGlyGlnGlnGlnTrpGlyAsp

TGGTCAATGTGATCGGCACCGGATTGGTATTGCGCCATCCGCAAACTGGGGAAGTGTGCTGGATTGTTTGTCTGGGTCAAGCCA
 TrpCysAsnValIleGlyThrGlyPheGlyIleArgProSerAlaAsnThrGlyAspSerLeuLeuAspSerPheValTrpValLysPro

GGGGGGAGTGTGACGGCACCAGCAGCAGTGGCCACGATTGACTCCCACTGTGCGCTCCAGATGCCTTGCAACCGGCGCTCAA
 GlyGlyGluCysAspGlyThrSerAspSerSerAlaProArgPheAspSerHisCysAlaLeuProAspAlaLeuGlnProAlaProGln

STOP
 GCTGGTGTGGTTCCAAGCCTACTTTGTGACGCTTCTCACAACGCAAAACCATCGTCTCTGTAAGGCTTTCGTGACCGGGCTTCAAAAC
 AlaGlyAlaTrpPheGlnAlaTyrPheValGlnLeuLeuThrAsnAlaAsnProSerPheLeu

Acc I
 AATGATGTGCGATGGTGTGGTTCGGGTTCCGGAGTCTTTGTCTACTTTGGTTGTCTGTCGAGGTCGGTAGACCGCAATGAGCAACT
 GATGGATTGTTGCCAGGATACTATAATTACATGGATGGTCTTTGTGATCAGTAGGCTAGAGAGAGAGAGAGAACATCTATCCACAAT

polyA. Bam HI
 GTCGAGTGTCTATT(A)₁₃GGGGATCC
 pUC8

Fig. 5

SUBSTITUTE SHEET

6/10

pUC8
CCCCCTATCTTAGTCCTCTTGTGTCCCAA

-----ENDO II signal sequence-----Kpn I

ATGGCGCCCTCAGTTACACTGCCGTTGACCACGGCCATCCTGGCNATTGCCGGGCTCGTCGCCGCCAGCAACCGGGTACLAGCACCCCC
MetAlaProSerValThrLeuProLeuThrThrAlaIleLeuAlaIleAlaArgLeuValAlaAlaGlnGlnProGlyThrSerThrPro 30

GAGGTCCATCCCAAGTTGACAACCTACAAGTGACAAAGTCCGGGGGGTGGCTGGCCAGGACACCTCGGTGGTCTTACTGGAAGTAC
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CGCTGGATGCACGACGCAAACTACAACCTCGTGACCGTCACCGCGGGCTCAACACCACGCTCTGCCCTGACGAGGGACCTGTGGCAAG
ArgTrpMetHisAspAlaAsnTyrAsnSerCysThrValAsnGlyGlyValAsnThrThrLeuCysProAspGluAlaThrCysGlyLys 90

Sal I
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SerGlyGlyTyrSerSerValSerProArgLeuTyrLeuLeuAspSerAspGlyGluTyrValMetLeuLysLeuAsnGlyGlnGluLeu 150

Sal I
AGCTTCGACGTCGACCTCTCTGCTCTGCCGTGGAGAGAAGCGCTCGCTCTACGTGTCTCAGATGGACGAGAACGGGGGCCCAACAG
SerPheAspValAspLeuSerAlaLeuProCysGlyGluAsnGlySerLeuTyrLeuSerGlnMetAspGluAsnGlyGlyAlaAsnGln 180

TATAACACGGCGGTGCCAACTACGGGAGCGGCTACTGCGATGCTCAGTGCCCGTCCAGACATGGAGGAACGGCACCCCTCAACACTAGC
TyrAsnThrAlaGlyAlaAsnTyrGlySerGlyTyrCysAspAlaGlnCysProValGlnThrTrpArgAsnGlyThrLeuAsnThrSer 210

Xho I
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HisGlnGlyGlnGlyPheCysCysAsnGluMetAspIleLeuGluGlyAsnSerArgAlaAsnAlaLeuThrProHisSerCysThrAla 240

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ThrAlaCysAspSerAlaGlyCysGlyPheAsnProTyrGlySerGlyTyrLysSerTyrTyrGlyProGlyAspThrValAspThrSer 270

AACACCTTCACCATCATCCCCAGTTCAACACGGACAACGGCTCGCCCTCGGGCAACCTTGTGAGCATCACCCGCAAGTACCAGXXXXX
LysGlnThrIleIleThrGlnPheAsnThrAspAsnGlySerProSerGlyAsnLeuValSerIleThrArgLysTyrGln..... 300

Sal I
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ValAspIleProSerAlaGlnProGlyGlyAspThrIleSerSerCysProSerAlaSerAlaTyrGlyGlyLeuAlaThrMetGlyLys 330

GCCCTGAGCAGCGCATGGTGTCTCGTGTTCAGCATTGGAAACGACAACAGCCAGTACATGAAGTGGCTCGACAGCGGCAACGGCGGCCCC
AlaLeuSerSerGlyMetValLeuValPheSerIleTrpAsnAspAsnSerGlnTyrMetAsnTrhLeuAspSerGlyAsnAlaGlyPro 360

Pst I
TGCAGCAGCAGCGAGGCAACCCATCCAACATCTGGCCAACAACCCCAACACGACGTCGTCTTCTCCAACATCCGCTGGGAGACATT
CysSerSerThrGluGlyAsnProSerAsnIleLeuAlaAsnAsnProAsnThrHisValValPheSerAsnIleArgTrpGlyAspIle 390

Sac I
GGGTCTACTACGAACTCGACT....x.....x..GAGCTCGACGACTTCGAGCAGCCGAGCTGCACGCAGACTCACTGGGGCAGTGC
GlySerThrThrAsnSerThr.....SerSerThrThrSerSerSerProSerCysThrGlnThrHisTrpGlyGlnCys 420

STOP
↓

GGTGGCATTGGGTACAGCGGGTGAAGACGTGCAGTGGGCACTACGTGCCAGTATAGCAACGACTACTACTCGCAATGCCTTTAGAGC
GlyGlyIleGlyTyrSerGlyCysLysThrCysThrSerGlyThrThrCysGlnTyrSerAsnAspTyrTyrSerGlnCysLeuxxx 450

Sac I
GTTGACTTGCCTCTGGTCTGTCCAGACGGGGCACGATAGAATCGGGCACGCAGGGAGCTCGTAGACATTGGGCTTAATATATAAGACA

POLY A | pUC 8
TGCTATGTTGTATCTACATTAGCAAATGACAAACAAATGAAAAAGAACTTATCAAGC(A)₂₅

Fig. 6

SUBSTITUTE SHEET

7/10

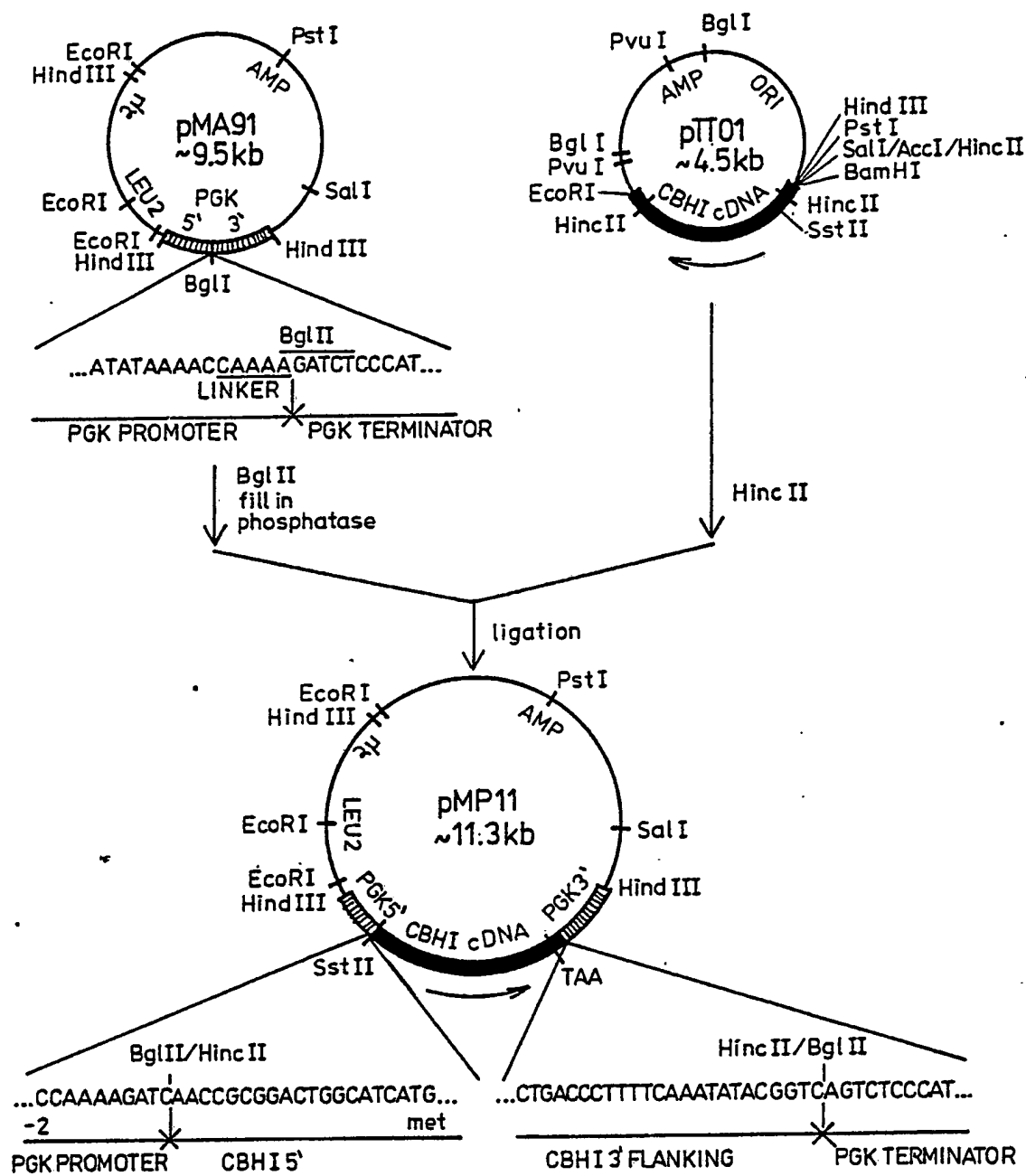


Fig. 7

8/10

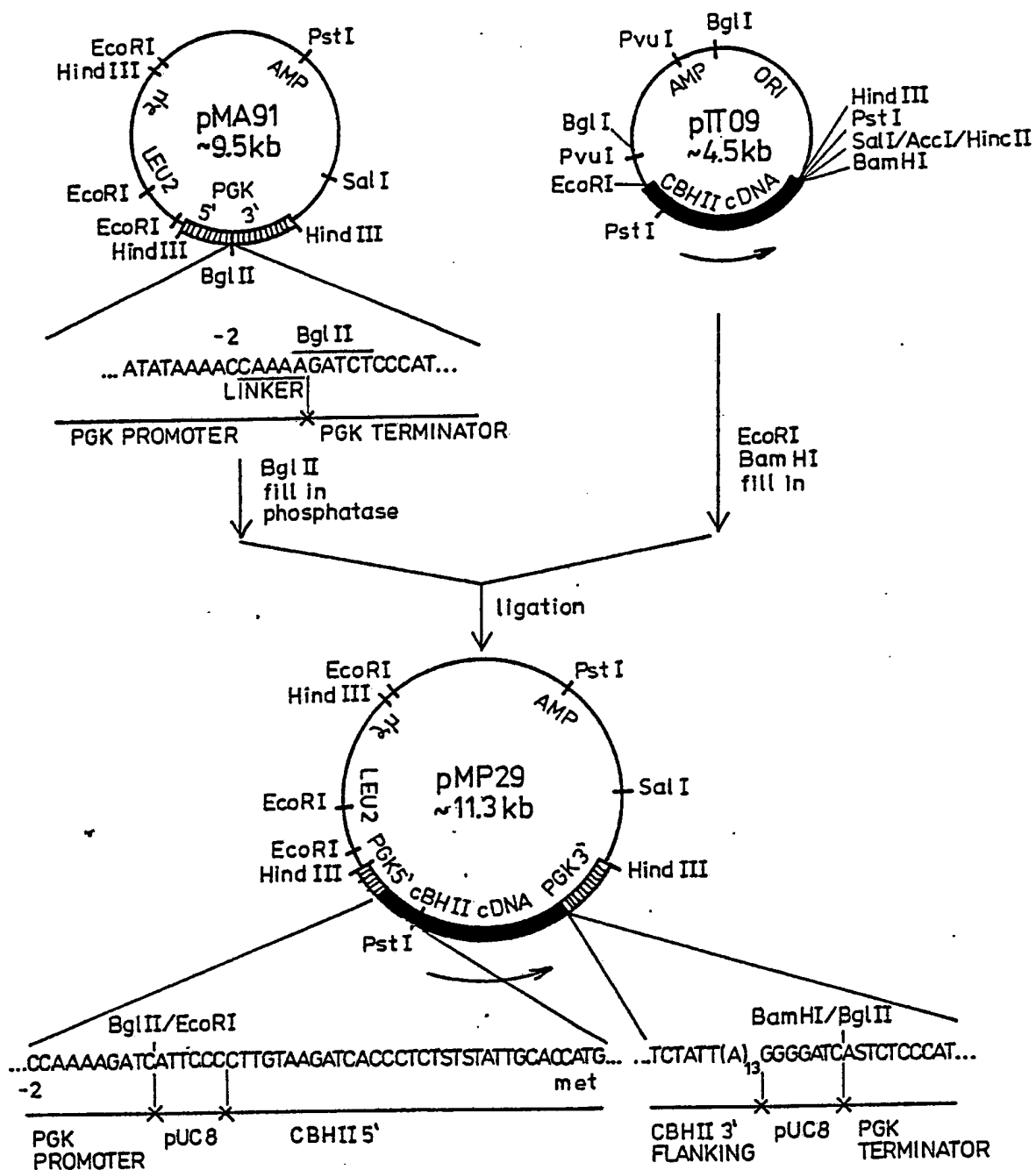


Fig.8

9/10

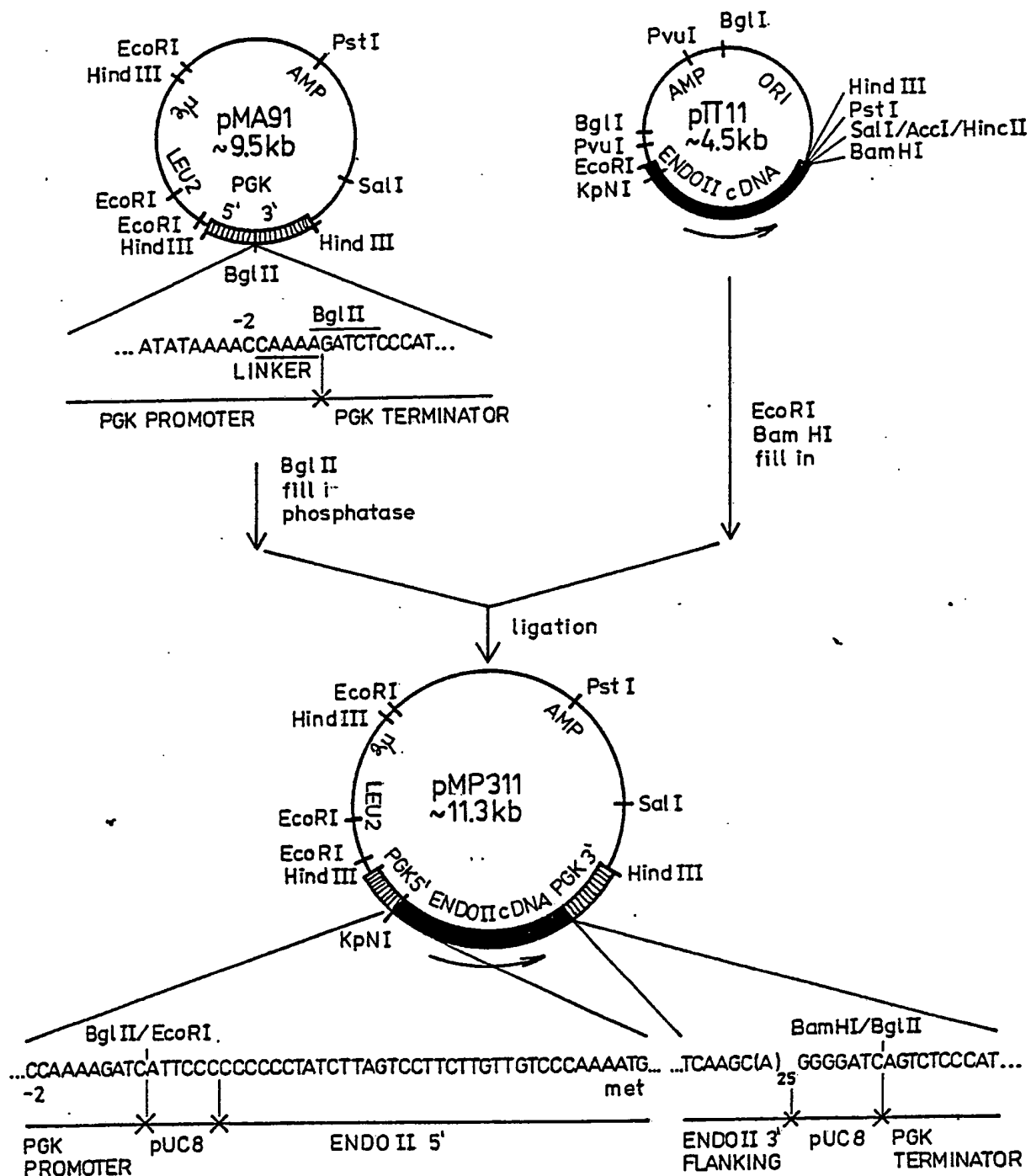
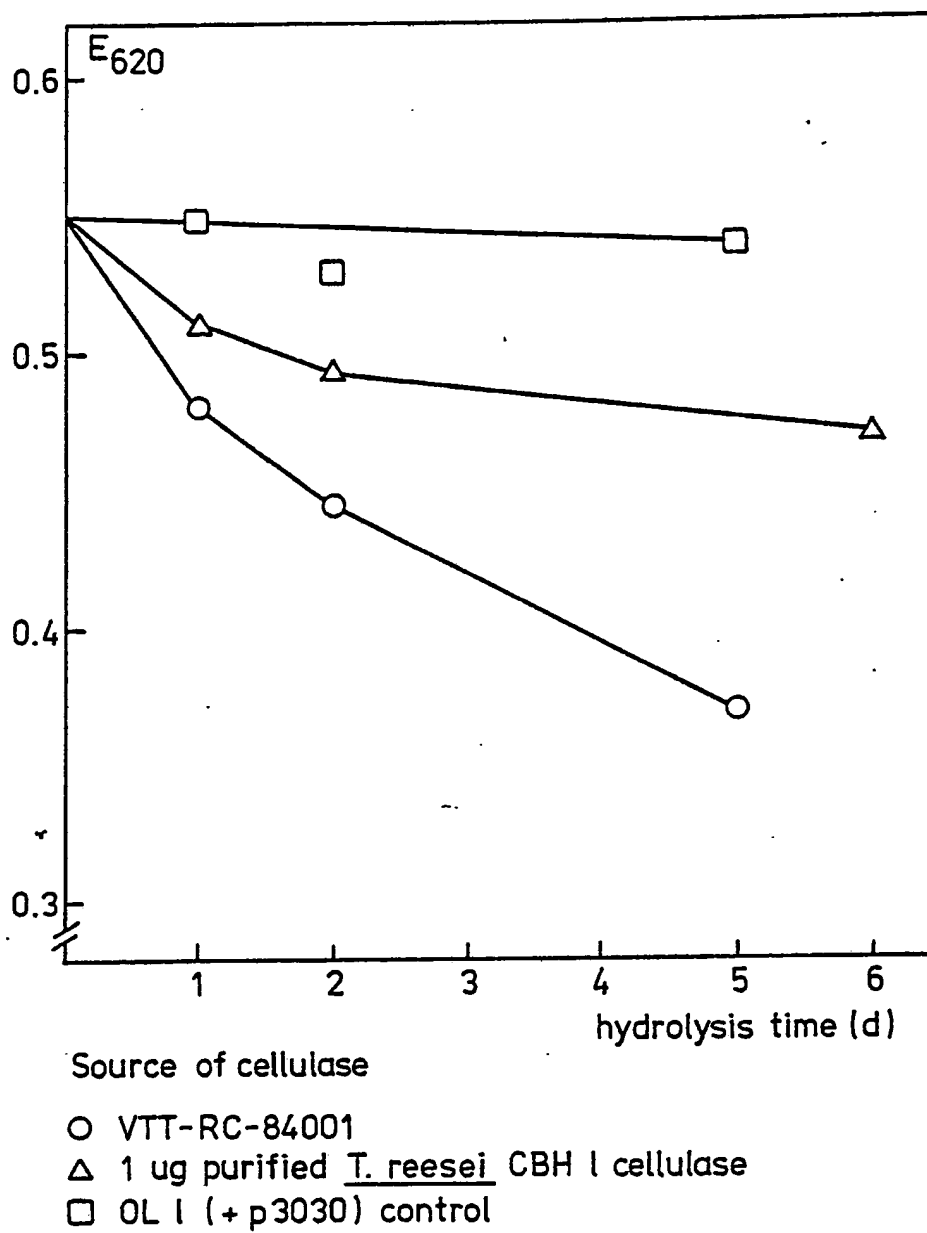


Fig.9

10/10

*Fig. 10*

INTERNATIONAL SEARCH REPORT

International Application No PCT/FI85/00039

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC ⁴ <div style="text-align: center; padding: 5px;">C 12 N 15/00, C 12 N 9/42</div>																	
II. FIELDS SEARCHED <div style="text-align: center; padding: 5px;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; padding: 5px;">Classification System</td> <td style="padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC 4</td> <td style="padding: 5px;">C 12 N 15/00; C 12 N 9/42</td> </tr> <tr> <td style="padding: 5px;">US C1</td> <td style="padding: 5px;">435:172, 317</td> </tr> </table> <div style="text-align: center; padding: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> <div style="padding: 10px;">SE, NO, DK, FI classes as above</div>			Classification System	Classification Symbols	IPC 4	C 12 N 15/00; C 12 N 9/42	US C1	435:172, 317									
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IPC 4	C 12 N 15/00; C 12 N 9/42																
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="padding: 5px;">1985-05-29</td> <td style="text-align: center; padding: 5px;">1985-06-12</td> </tr> <tr> <td style="padding: 5px;">International Searching Authority</td> <td style="padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="padding: 5px;">Swedish Patent Office</td> <td style="text-align: center; padding: 5px;"> Inga-Karin Petersson </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	1985-05-29	1985-06-12	International Searching Authority	Signature of Authorized Officer	Swedish Patent Office	 Inga-Karin Petersson							
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A2, 0 088 632 (GENENTECH, INC) A3, 14 September 1983 & GB, 2116567 FR, 2523152 AU, 12152/83 JP, 58174396 DE, 3308215	15, 19, 20-30

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